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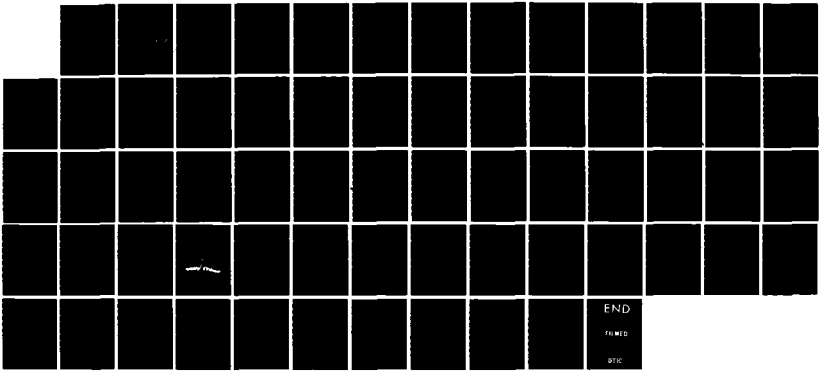

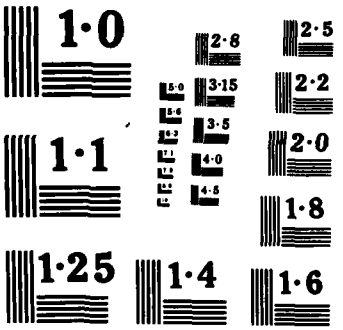
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GALACTOSE OXIDASE IN STEREOSPECIFIC OXIDATION OF PRIMARY ALCOHOLS

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GALACTOSE OXIDASE IN STEREOSPECIFIC OXIDATION OF PRIMARY ALCOHOLS

A Thesis

by

ROBERT LEE ROOT

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May 1985

ABSTRACT

Galactose Oxidase in Stereospecific Oxidation of Primary Alcohols.

(May 1985)

Robert Lee Root, B.S., United States Military Academy

Chairman of Advisory Committee: Dr. Chi-Huey Wong

The stereospecificity of oxidation of several polyols and substituted polyols by the enzyme galactose oxidase was studied. An enzymatic method involving aldehyde dehydrogenase, D- and L-lactate dehydrogenase, and alcohol dehydrogenase for the determination of optical purity of the oxidation product was used. Polyols and substituted polyols of three, four, five, and six carbons were tested as substrates using a horseradish peroxidase-chromagen coupled assay. A minimum structure required for polyol substrates of galactose oxidase was developed. Galactose oxidase was used in an attempt to synthesize D-threose, L-xylose, L-galactose, and L-glucose. Reasons for poor yields in three of the four syntheses were discussed.

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It is with my sincerest appreciation that I thank my advisor, Dr. Chi-Huey Wong, for his help, instruction, and patience. He has taken me from total ignorance of enzymes to a continually-growing working knowledge of enzyme chemistry. While I still have plenty to learn, Dr. Wong has helped me on the way.

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My wife, Robin, deserves the most thanks, for putting up with me these last couple of years, especially, my first semester. Without her loving support, I would have had a very difficult time completing my work.

Finally, I would like to thank the Robert A. Welch Foundation and the National Science Foundation for their generous support.

DEDICATION

To my mother, Lee,

who hoped I could make a sugar diabetics could use,

and

my wife, Robin,

because of her love.

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CHAPTER III

PRODUCT STEREOCHEMISTRY

Introduction

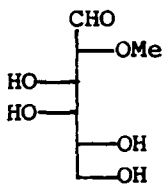
Klibanov's method of determining the optical purity of the product of his glycerol oxidation is not infallible. He reports 100% enantiomeric excess based on the specific rotation of his product, -9.3° (33). The reported values of the specific rotation of L-glyceraldehyde range from -8.5° to -11° with -8.7° most often used (34). Since there is disagreement on the reported value, a better method of determining the stereochemical outcome of the experiment is needed.

Although Klibanov predicted (R)-3-chloro-1,2-propanediol would be a substrate and showed this to be the case, he had no way of showing (S)-3-chloro-1,2-propanediol was not a substrate (33). Once again his claim of enantiomeric selectivity is suspect. Because of the results of Klibanov's experiment and the lack of reliability of his method, an enzymatic method for verifying the stereochemical products has been developed.

Results

Klibanov's experiment was repeated with a variety of possible substrates. Initially, glycerol and racemic 3-chloro-1,2-propanediol were used to test the enzymatic method and to reproduce and verify Klibanov's results. Several other compounds were then tested to

was oxidized, but he could not check the S-form because a satisfactory method of synthesis was not available (33).



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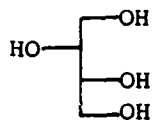

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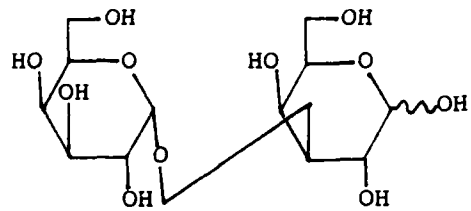

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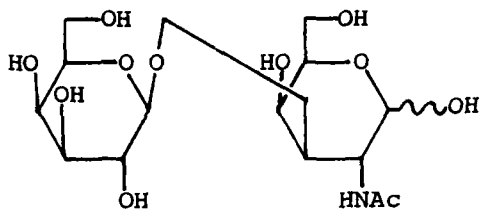

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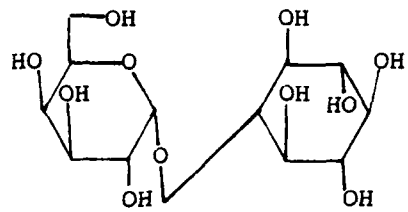

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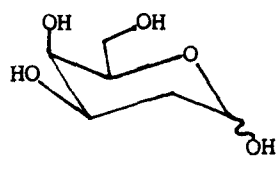

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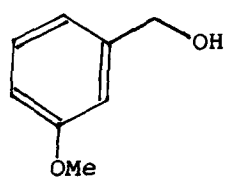

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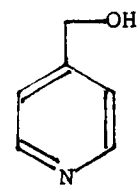

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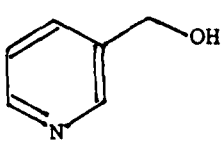

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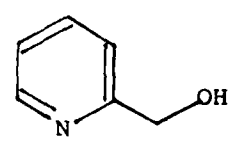

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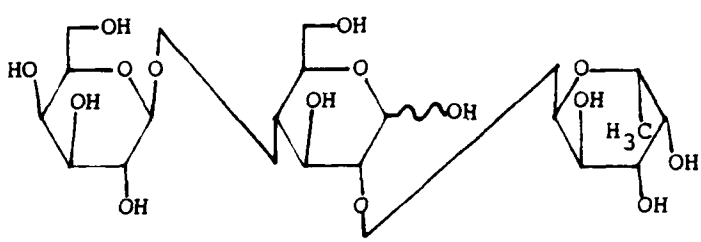

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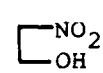

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Figure 5 (Continued).

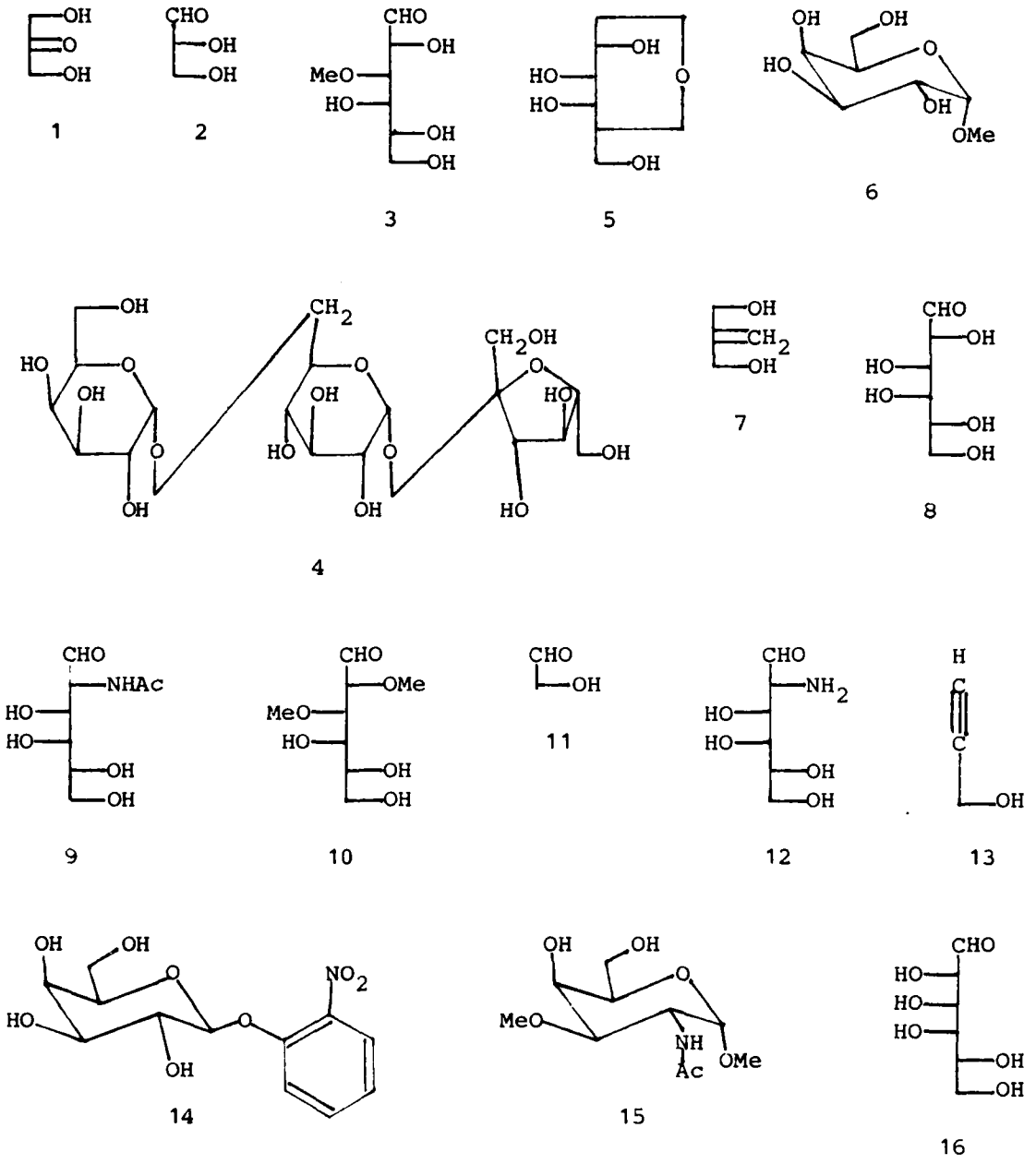

Figure 5. Substrates of galactose oxidase.

TABLE 1 Continued

Substrate	Relative Reactivity	Reference
2-O-Methyl-D-galactose, 17	50	26
D-Threitol, 18	50	27
O-α-D-Galactopyranosyl-(1→3)-D-galactose, 19	48	26
O-β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactose, 20	39	26
1-O-(α-D-Galactopyranosyl)-myo-inositol, 21	37	26
2-Deoxy-D-galactose, 22	32	25
m-Methoxybenzyl alcohol, 23	32	18
Glycerol, 24	20	27
p-Pyridine carbinol, 25	14.3	18
m-Pyridine carbinol, 26	13.5	18
Hydroxyacetophenone, 27	4.8	18
Acetol, 28	4.75	18
2'-O-(α-D-Fucopyranosyl)-lactose, 29	4	26
2-Propene-1-ol, 30	3.5	18
o-Pyridine carbinol, 31	3.2	18
2-Nitroethanol, 32	1.6	18

TABLE 1

Relative Reactivities of Galactose Oxidase Substrates

Substrate	Relative Reactivity	Reference
1,3-Dihydroxyacetone, 1	377	18
D-Glyceraldehyde, 2	301	18
3-O-Methyl-D-galactose, 3	172	26
Guaran[a]	150	26
Raffinose, 4	145	26
1,5-Anhydro-D-galactitol, 5	134	26
Methyl-α-D-galactopyranoside, 6	118	26
2-Methylene-1,3-propanediol, 7	112	18
D-Galactose, 8	100.00	
N-Acetyl-D-galactosamine, 9	92	25
Ovomucoid[b]	87	26
2,3-Di-O-methyl-D-galactose, 10	76	26
Glycolaldehyde, 11	75.1	18
D-Galactosamine, 12	75	25
Propargyl alcohol, 13	59.5	18
o-Nitrophenyl-β-D-galactopyranoside, 14	57	26
Methyl-2-acetamido-2-deoxy-3-O-methyl-α-D-galactopyranoside, 15	54	26
D-Talose, 16	52	27

[a] Guaran is a polysaccharide consisting of β-(1→4)-mannopyranosyl linkages with side-chain α-(1→6)-galactopyranosyl linkages to half of the mannosides (28).

[b] Ovomucoid is a glycoprotein with approximately 5% galactoside content (29).

Numerous studies (18,25-27) have determined that galactose oxidase catalyzes the oxidation of many primary alcohols. Table 1 lists those alconols (Figure 5) which have relative reactivities of 1 or greater (D-galactose = 100) (28,29). A variety of methods were used to measure the reactivities, so the relative rates are only qualitative.

Currently, galactose oxidase is used mainly as an analytical tool. Its preference for D-galactose and D-galactopyranosides make it an excellent agent for determining the presence of these compounds in the blood (30) and the presence of galactosyl residues in polysaccharides (31). Very few attempts to use the enzyme in synthesis have been reported.

Most enzymes exhibit not only substrate specificity, but chiral and prochiral specificity as well (1). It is not unlikely, therefore, to expect galactose oxidase to have the same properties. D-Galactose may lose either of two protons from C-6 to form the aldehyde. These protons are prochiral, and the enzyme would be expected to remove one of them preferentially. Experiments by Maradufu, Cree, and Perlin using D-galactose deuterated at C-6 to give the S- and R-form showed this to be the case (32); galactose oxidase removes the pro-S proton.

Galactose oxidase has also been shown to exhibit prochiral and enantiomeric stereospecificity with substrates (33). Klibanov and co-workers used galactose oxidase to catalyze the oxidation of glycerol to L-glyceraldehyde. He then used this result to predict that (R)-3-chloro-1,2-propanediol would be oxidized by the enzyme while its stereoisomer would not. His prediction was correct in that the R-form

reoxidizes the Cu(I) to Cu(II) with the loss of H_2O_2 (23). Hamilton's other mechanism requires copper in a trivalent state in its active form (Figure 3) (12). He claims that Cu(II) is actually the inactive

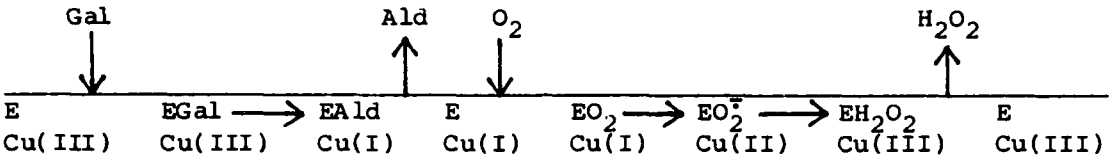

Figure 3. Hamilton's Cu(I)-Cu(II)-Cu(III) ping-pong mechanism (12).

form of the enzyme, and it must be oxidized to Cu(III) before it becomes active. The enzyme cycles from Cu(III) to Cu(I) and back to Cu(III) during turnover. Again the mechanism is based on ping-pong kinetics.

Kosman and co-workers, however, have proposed a mechanism based on a kinetically sequential reaction (Figure 4). Galactose oxidase

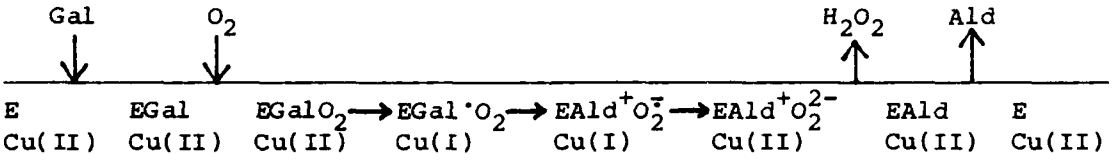

Figure 4. Kosman's sequential kinetic mechanism of galactose oxidase (18,24).

first binds D-galactose followed by O_2 to give tne enzyme-reactant complex (24). The complex then undergoes rate-determining proton abstraction and reduction of Cu(II) to Cu(I) followed by rapid oxidation of the substrate free radical and oxidation of tne Cu(I) back to Cu(II) (18). The products are then predicted to be released with hydrogen peroxide first and the aldehyde last (24).

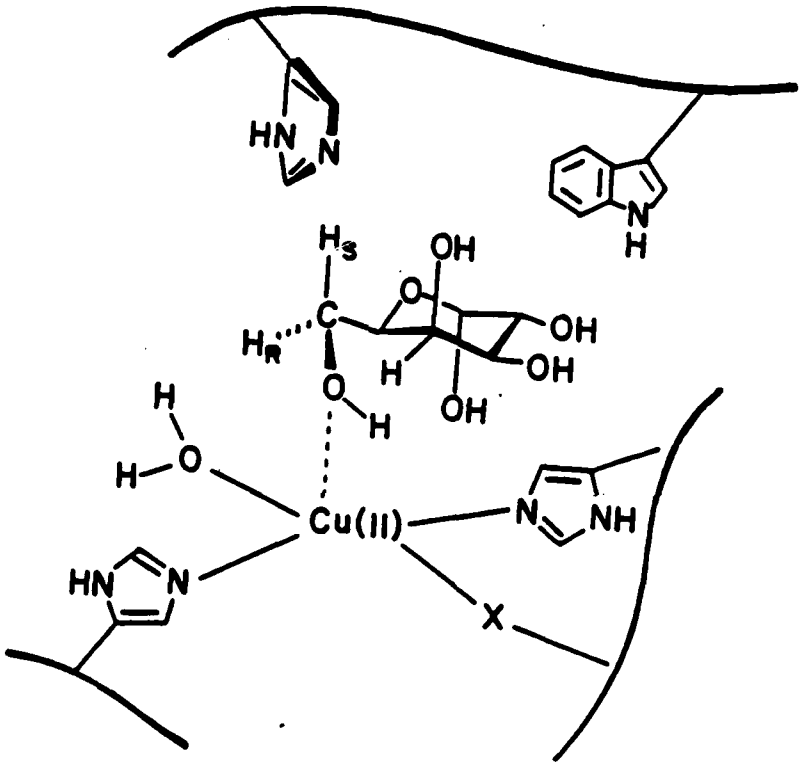

Figure 2. The current concept of the active site of galactose oxidase (18).

presence and importance of the histidine residue was demonstrated by pH profiles and carboxymethylation (22). Only one of the eight histidines is alkylated by iodoacetamide, and the activity is lost. Treatment of the enzyme with N-bromosuccinimide oxidizes up to four tryptophan residues, however activity is reduced to 2% by oxidation of only two residues. Ettinger and co-workers have speculated that there are two similarly reactive Trp residues, but only one is associated with the active center (17). The role of the tryptophan is unclear, but its location 12-15 Å from the copper indicates it may have a very important bearing on access to the Cu(II) or in maintaining the conformation of the active site (18). Ettinger and Kosman have used the results of these experiments and others to develop a concept of the galactose oxidase active site (Figure 2) (18).

The exact mechanism of galactose oxidase is not known. It is known to be a Bi Bi mechanism since it has two substrates and two products. There has been considerable disagreement about the order of reaction. Hamilton and co-workers have proposed two mechanisms based on ping-pong kinetics (12,23). His earlier mechanism is shown in Figure 1. This mechanism maintains that Cu(II) is converted to Cu(I)

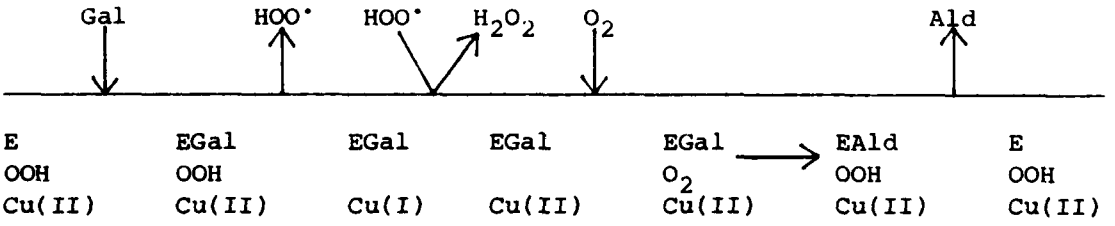

Figure 1. Hamilton's Cu(I)-Cu(II) ping-pong mechanism (23).

with the loss of a superoxide radical which then immediately

CHAPTER II

BACKGROUND

Galactose oxidase was discovered in 1959 by Cooper and co-workers (13) and has been variously described as being produced by Polyporus circinatus (13), Dactylium dendroides (14), and more recently, Gibberella fujikuroi (15). The enzyme is a single-chain protein of molecular weight 68,000 daltons (16). The amino acid composition includes 8 histidine, 18 tryptophan, and 5 cysteine residues (17). Of the five Cys, only one is titratable as free -SH, and only if the copper has been removed from the enzyme (18). Galactose oxidase is an unusual enzyme in that it is the only known enzyme containing one Type 2 Cu(II) with no other prosthetic groups, and it catalyzes a two-electron redox reaction using only a single Cu(II) (16).

The active site residues and geometry have not yet been positively determined. It is known that the copper atom is located at the active site; its removal gives an inactive apoenzyme (19). Electron spin resonance (ESR) studies indicate that the copper has a pseudo-square-planar geometry (20,21). Ettinger and Kosman's ESR studies reveal two of the equatorial ligands to be imidazole nitrogens, one of the other equatorial sites is H_2O or OH^-, and the last site is still unknown (18). The axial site of the resting enzyme is expected to have a H_2O ligand as indicated by its accessibility to F^- (18).

Other important amino acid residues in the vicinity of the active site include one nonligand histidine and one tryptophan (18). The

synthesize unusual sugars are reported.

of their suspected high carcinogenicity (8). Saccharin, although still accepted as "safe," is suspected of being a cancer-causing agent (9), and it leaves a bitter aftertaste. Aspartame is the methyl ester of aspartylphenylalanine. While aspartame is not known to be a carcinogen, high blood levels of phenylalanine, one of the metabolic products of aspartame, is associated with mental retardation (10). It has been suggested that the unusual L-sugars could resolve all the problems associated with dietetic sweeteners (6c).

Two general chemical methods have recently been developed for the synthesis of unusual L-sugars. One is an asymmetric Diels-Alder reaction (11), and the other is based on asymmetric epoxidation (4). The former procedure involves a chiral auxiliary, silylation, a lanthanide chiral reagent for a Lewis acid-catalyzed Diels-Alder reaction, and hydroxylation. The latter involves chain extension, asymmetric epoxidation, base-catalyzed epoxide ring-opening, and oxidation. Typically, there have not been any attempts to use enzymes in the synthesis of L-sugars.

This paper reports the first attempt to synthesize L-sugars by the use of the enzyme, galactose oxidase (D-galactose: O_2 oxidoreductase; EC 1.1.3.9), which catalyzes the following reaction:

$$\text{D-galactose} + O_2 \longrightarrow \text{D-galactohexodialdose} + H_2O_2 \quad (12).$$

Included in this report are experiments, which were used to determine and confirm the stereochemical outcome of the oxidation, and substrate tests used to identify which polyols and substituted polyols could be used in the syntheses. Finally, the results of four attempts to

CHAPTER I

INTRODUCTION

During the last several years there has been an increasing interest in the synthesis of asymmetric organic compounds. One synthetic method which is often overlooked by most organic chemists is the use of enzymes as catalysts. Reasons for this include unfamiliarity, perceived technical difficulties, lack of generality, and tradition (1). Enzymes are custom-made for asymmetric syntheses. In many cases, enzymes can perform, in one step, a desired stereospecific transformation which might require several steps using conventional chemical methods (1).

A considerable amount of the interest in asymmetric synthesis has centered on carbohydrates and carbohydrate derivatives, such as antibiotics (2) and unnatural sugars (3-5). Since the majority of natural sugars occur in only the D-form, unnatural sugars, or L-sugars, are understandably expensive. Despite their expense, they are important because of their potential for use as precursors for many natural products and especially as safe, non-caloric sweeteners (6,7).

For many years the only types of dietetic sweeteners available were cyclamates and saccharin. More recently, aspartame has become widely used. All of these sweeteners have undesirable characteristics. In 1969 all types of cyclamates were removed from the Food and Drug Administration's "Generally Recognized As Safe" list because

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This thesis follows the style and format of Bioorganic Chemistry.

confirm the stereochemical outcome of galactose oxidase oxidation.

The general scheme of the enzymatic method is to use the specificity of enzymes in assays to measure the concentrations of L- or D- products. Substrates are identified by assaying an aliquot of the reaction mixture with aldehyde dehydrogenase (aldehyde: $NAD(P)^+$ oxidoreductase, EC 1.2.1.5). If the assay is positive, the entire reaction product is then converted to its corresponding carboxylic acid by using aldehyde dehydrogenase. The acid is then divided and tested by L-(+)-lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase, EC 1.1.1.27) and D-(−)-lactate dehydrogenase (D-lactate: NAD^+ oxidoreductase, EC 1.1.1.28), respectively. The concentrations obtained from these assays can be used to calculate the enantiomeric excess according to Equation 1. If the aldehyde product is not a

$$e.e. = \frac{[L] - [D]}{[L] + [D]} \qquad (1)$$

substrate of aldehyde dehydrogenase, the optically pure alcohol must be used as the starting material and the product tested with alcohol dehydrogenase (alcohol: NAD^+ oxidoreductase, EC 1.1.1.1). The substrates tested by this method and their optical purities are listed in Table 2 (Figure 6).

Discussion

It is apparent from these results that Klibanov was correct in claiming 100% enantiomeric excess. As expected, the product from oxidation of glycerol is L-glyceraldehyde, thus refuting the claim by

TABLE 2

Stereochemical Results of Oxidation by Galactose Oxidase

Substrate[a]	Product[b]	Enantiomeric Excess (%)
Glycerol, 24	L-(—)-glyceraldehyde, 33	100
3-Chloro-1,2-propanediol, 34	(R)-3-Chloro-2-hydroxypropanal, 35	100
Propylene glycol, 36	No reaction	
α-O-Benzyl glycerol, 37	L-α-O-Benzyl glyceraldehyde, 38	100
(S)-(+)-1,3-Butanediol, 39	No reaction	
(R)-(—)-1,3-Butanediol, 40	No reaction	

[a] All substrates are racemic unless otherwise indicated.

[b] Identity of products based on known results of oxidation by galactose oxidase and stereochemistry based on enzymatic assay.

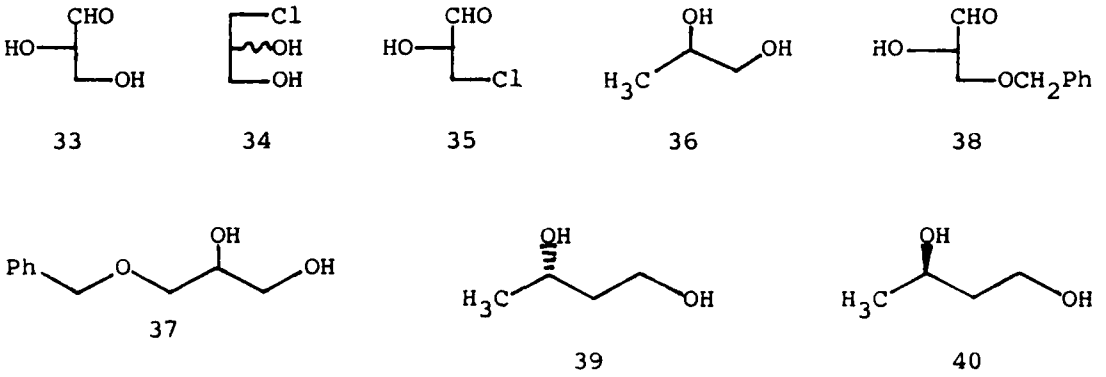

Figure 6. Compounds tested using enzymatic stereochemical determination.

Knull and co-workers that the product is D-glyceraldehyde (27). By using the enzymatic assay method to determine optical purity, the product of the oxidation of 3-chloro-1,2-propanediol was confirmed; (S)-3-chloro-1,2-propanediol is not a substrate.

Based on the results obtained from the first two reactions, it was predicted that if propylene glycol and O-benzyl glycerol were substrates, they would give L-aldehydes. In the case of O-benzyl glycerol, the L-product was obtained. The assay of the propylene glycol reaction mixture with alcohol dehydrogenase indicated no aldehyde present. This was unexpected since it has been reported that propylene glycol is a substrate (18). It could only be assumed that it is a very poor substrate, and the reaction had not been allowed to continue for sufficient time to build concentrations of product high enough to be observed by the assay.

To determine if the substituent at the number two carbon was important, the optically pure isomers of 1,3-butanediol were tested. No aldehyde product was indicated by alcohol dehydrogenase assay. The enzyme apparently requires a substituent larger than a proton at the carbon α to the aldehyde product.

Since all tests using D-lactate dehydrogenase were negative, it was not necessary to determine the concentrations of the products to determine optical purity. It was sufficient to insure that the product reacted with L-lactate dehydrogenase.

Conclusion

Galactose oxidase catalyzes a stereospecific reaction. When

using a diol substrate, the enzyme will act to give exclusively the L-form of the aldehyde (Figure 7). All three compounds which gave positive tests as substrates also gave stereochemical results consistent with this prediction.

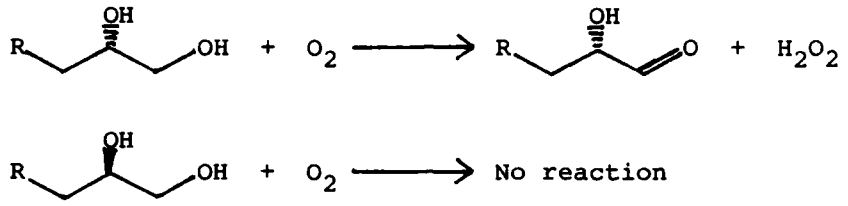

Figure 7. Predicted stereochemical results of oxidation by galactose oxidase.

CHAPTER IV

SUBSTRATE TESTING

Introduction

In order to make his prediction, Klibanov used the configuration of D-galactopyranose (33). This seems to be a good indicator. Consider the configuration of D-galactose as it appears in the current concept of the galactose oxidase active site (Figure 2). By testing numerous other compounds and by adapting Klibanov's example of D-galactopyranose, a more generalized structure has been developed to predict possible substrates and their products of oxidation.

Substrate testing by Klibanov's method is too slow and too insensitive to be effective, and it gives no indication of reaction rate. The horseradish peroxidase-chromagen assay is ideally suited for these purposes (35). While there are several reports (18,23,26) indicating that this method suffers from serious deficiencies, they all note that the assay is quite sensitive and very useful unless detailed kinetic analyses are required. For the purposes of substrate identification, however, the assay is adequate.

Results

Polyols and substituted polyols of three, four, five, and six carbons were tested as substrates. Initially, the choice of compounds was limited to three-carbon or four-carbon species. Table 3 (Figure 8) lists the results of these preliminary tests.

TABLE 3

Relative Velocities of Three- and Four-Carbon Polyols

Substrate	Relative Velocity[a]	Condition[b]
Glycerol, 24	100.00	
(S)-1,2-Propanediol, 41	2.4	c
(R)-1,2-Propanediol, 42	0	c
3-Chloro-1,2-propanediol, 34	36.6	d
DL-α-O-Benzyl glycerol, 37	12	e
DL-α-O-Benzyl glycerol, 37	56	f
2-Methyl-1,3-propanediol, 43	1.76	d
2-Amino-1,3-propanediol, 44	0	c

[a] Velocities are qualitative since the measurements were taken under various conditions. Glycerol was arbitrarily set equal to 100.00.

[b] Glycerol was tested at a variety of conditions, and the relative velocities were either determined directly against glycerol or extrapolated from glycerol results.

[c] Substrate 0.100 M, commercial enzyme preparation 25 µg/mL.

[d] Substrate 0.400 M, commercial enzyme preparation 6.25 µg/mL.

[e] Substrate 42.7 mM, commercial enzyme preparation 8.3 µg/mL.

Substrate 42.7 mM, commercial enzyme preparation 8.3 µg/mL, 4.34 mM $K_3Fe(CN)_6$.

TABLE 3 Continued

Substrate	Relative Velocity[a]	Condition[b]
L-Threitol, 45	0	g
D-Threitol, 18	1500	g
Erythritol, 46	0	g
(S)-1,3-Butanediol, 39	0	g
(R)-1,3-Butanediol, 40	0	g
3-Bromo-1,3-propanediol, 47	63.3	h
3-Fluoro-1,3-propanediol, 48	3.8	h
3-Butene-1,2-diol, 49	12.5	h
D-Glyceraldehyde, 2	1080	g

[g] Substrate 50 mM, commercial enzyme preparation 8.3 µg/mL.

[h] Substrate 0.100 M, commercial enzyme preparation 8.3 µg/mL.

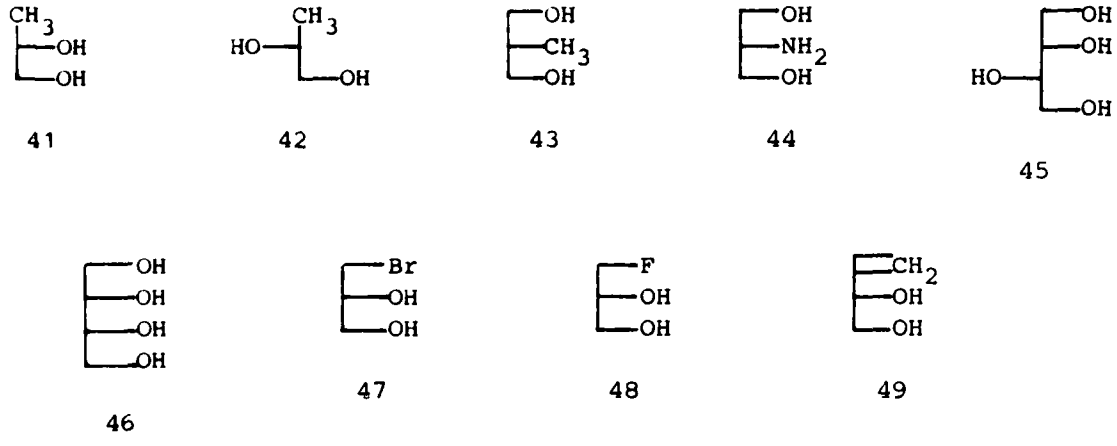

Figure 8. Three- and four-carbon compounds tested as substrates.

Having completed the studies on three- and four-carbon polyols, an attempt was made to identify a minimum structure necessary to be a substrate for galactose oxidase. Klibanov's method was adapted to develop the structure used to predict probable substrates (Figure 9).

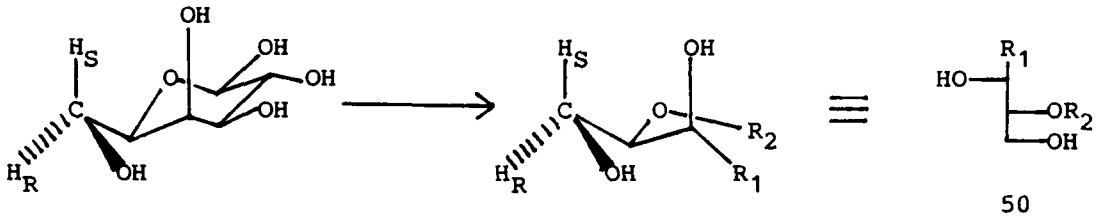

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Figure 9. Development of minimum required polyol substrate structure.

The minimum structure was used to predict the following as possible substrates: xylitol, D-arabitol, galactitol, and L-glucitol. In each case the terminal three carbons can be written in the form of 50. Structure 50 was also used to predict that the following compounds would not be substrates: L-xylose, L-lyxose, D-arabinose, D-ribose, L-galactose, D-glucose, and L-glucose. In each case the three terminal carbons cannot be written in the form of 50. The results of the substrate testing of these compounds are given in Table 4 (Figure 10).

Discussion

Glycerol is the ideal substrate to use as the reference because its identity as a substrate of galactose oxidase is well-documented (27,33), and the stereochemical outcome of the reaction is known (33, vide infra). Using the results of the substrate testing and the stereochemical products discussed previously, a minimum structure of

TABLE 4

Relative Velocities of Five- and Six-Carbon Polyols

Substrate	Relative Velocity[a]	Condition[b]
Glycerol, 24	100.00	
Xylitol, 51	550	c
D-Arabitol, 52	76.9	c
Galactitol, 53	2.5	c
Galactitol, 53	36.3	d
L-Glucitol, 54	3.3	e
L-Xylose, 55	0	c
L-Lyxose, 56	0	c
D-Arabinose, 57	0	c
D-Ribose, 58	0	c
L-Galactose, 59	0	c
D-Glucose, 60	0	c
L-Glucose, 61	0	c

[a] Velocities are qualitative since the measurements were taken under various conditions. Glycerol was arbitrarily set equal to 100.00.

[b] Glycerol was tested at a variety of conditions, and the relative velocities were either determined directly against glycerol or extrapolated from glycerol results.

[c] Substrate 50 m$\underline{M}$, commercial enzyme preparation 8.3 µg/mL.

[d] Substrate 0.100 $\underline{M}$, commercial enzyme preparation 0.100 mg/mL, 0.050 m$\underline{M}$ $K_3Fe(CN)_6$.

[e] Substrate 0.167 $\underline{M}$, commercial enzyme preparation 0.175 mg/mL, 0.050 m$\underline{M}$ $K_3Fe(CN)_6$.

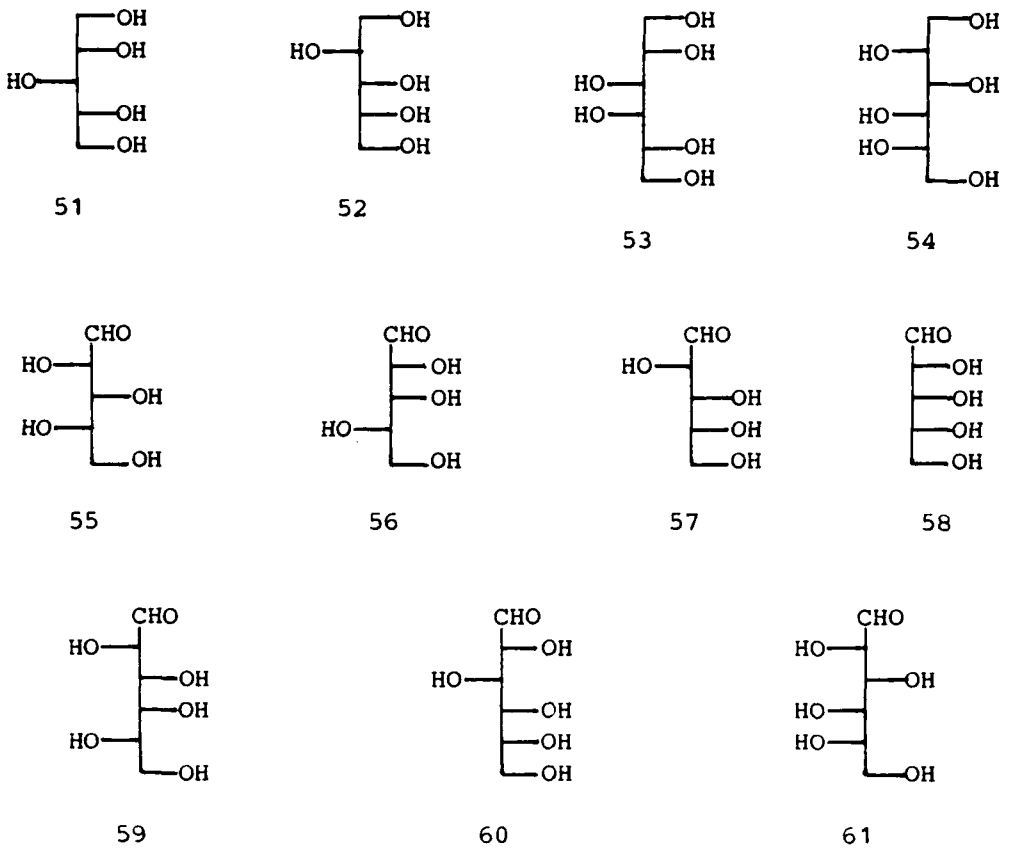

Figure 10. Five- and six-carbon compounds tested as substrates.

polyol substrates has been developed, and the stereochemistry can be predicted. Each of the compounds tested and its importance will be discussed in this section.

When propylene glycol was tested using Klibanov's reaction method and the enzymatic determination method, it was not indicated as a substrate. This appeared to be in contradiction of previously reported results (18). The chromagen assay indicates that propylene glycol is a substrate, but only the S-enantiomer. The relative slowness of the reaction could explain the anomaly. It should be pointed out that propylene glycol exhibited an extremely long induction period of the type described by Hamilton et al. (23). The induction period was over an hour when the reaction was run at room temperature. Under conditions of Klibanov's reaction, 4 ^{o}C, the induction period could have been substantially longer. The importance of the positive reaction is that the absence of a group in the position corresponding to the axial hydroxyl in D-galactose reduces the reaction rate considerably. Note, however, that the product, (S)-2-hydroxypropanal would be the L-product.

The testing of the 3-halo-1,2-propanediols also gives some insight about the position corresponding to the axial hydroxyl in D-galactose. The results indicate that the reaction rate varies according to increasing size since the rate increases from fluorine to bromine. This appears to contradict Maradufu and Perlin who report exactly the opposite effect (36). However, they used 4-deoxy-4-halo derivatives of D-galactose in which the four position was fixed. The substrates tested here were capable of free rotation about the C-3:C-2

bond, which might indicate an increase in rate with decreasing electron withdrawing inductive effect, instead. Again the expected products would be the L-3-halo-2-hydroxypropanals.

The low activity of 2-methyl-1,3-propanediol and the lack of activity with 2-amino-1,3-propanediol indicate that galactose oxidase prefers an oxygen group in the C-5 position of D-galactose over amino or methyl groups. The oxygen group does not have to be at the alcohol oxidation level as shown by the strong reaction with 1,3-dihydroxyacetone (18). The general preference of the enzyme for galactosides indicates that either a hydroxy or an ether linkage are acceptable at the carbon adjacent to the carbon being oxidized. Although the product of the oxidation of the 2-methyl-1,3-propanediol reaction was not isolated, it was expected to be (S)-2-methyl-3-hydroxypropanal based on the earlier derived stereochemistry (vide infra).

Because the slow reaction rate of propylene glycol caused its reaction to be missed using Klibanov's method, both enantiomers of 1,3-butanediol were retested with the assay method. The inactivity of these compounds again indicated the importance of the substituent at the C-5 position of D-galactose. The absence of a functional group at C-5, preferably OH or OR, severely hinders the enzyme.

The testing of L-threitol, erythritol, and D-threitol (45, 46, and 18, respectively) provided the evidence required to complete the minimum structure prediction model (Figure 9). L-threitol and D-threitol are enantiomers, and erythritol is a diastereomer of both. In each of the cases for which the enzymatic assay was used to check

the stereochemistry of the galactose oxidase reaction, the products were isomeric at the carbon α to the aldehyde. The selectivity of D-galactose over D-glucose (25) and the selectivity of D-threitol over L-threitol and erythritol indicate that substrates will be isomeric at the carbon β to the aldehyde product. The results reported here allow the generalization shown in Figure 11 about reactions catalyzed by galactose oxidase.

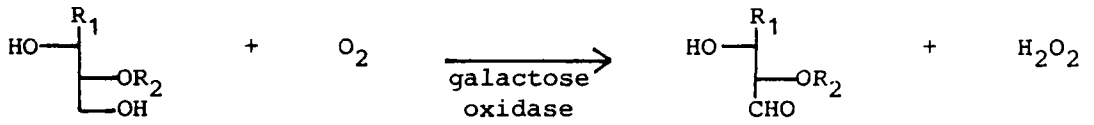

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Figure 11. Expected stereochemical outcome of oxidation of polyols of form 50 by galactose oxidase.

Having predicted xylitol, D-arabitol, galactitol, and L-glucitol to be substrates of galactose oxidase by using the model, it was disappointing to find that galactitol had already been determined to be an extremely weak substrate (25). L-Glucitol was initially found to be completely unreactive. However, later it was shown to be a very weak substrate. Since galactitol is simply the reduced form of D-galactose, it seems unusual that it is not more reactive. It is also puzzling that L-glucitol is not more reactive. The four- and five-carbon polyols with the correct configuration are very reactive. The only apparent explanation which can be offered at this time is that the enzyme active site is shaped so that a ring form is required for substrates containing six or more carbons. Since galactitol and L-glucitol can only attain such a configuration with considerable

crowding, their reactivity may be restricted by the unfavorable conformational equilibria. This hypothesis is supported, in part, by the very high reactivity of 1,5-anhydro-D-galactitol (25,26).

When galactitol and L-glucitol were found to be so unreactive, an attempt to make the reactions more favorable was made. Hamilton and co-workers had reported that the presence of small concentrations of $K_3Fe(CN)_6$ significantly increases the rate of the galactose oxidase reaction (12,37,38). The addition of 50 μM $K_3Fe(CN)_6$ did, in fact, increase the reaction rates of galactitol and L-glucitol to reasonable rates. It was reported by Singh and co-workers (39) that $K_3Fe(CN)_6$ oxidizes galactitol to its dicarboxylic acid. However, their reaction is done under extremely alkaline conditions and with much more concentrated oxidant. Under conditions of the assay (5 mM phosphate buffer, pH 7.0) the oxidation of galactitol by $K_3Fe(CN)_6$ does not proceed at a rate sufficient to account for the change in absorbance observed in the galactose oxidase reaction. Potassium ferricyanide also oxidizes o-dianisidine, the chromagen used in the assay, but again, at the concentrations used in the assay, the increase in absorbance is not enough to account for the large increases seen in the enzyme reaction. Increases in galactitol concentration appear to affect the reaction rate in a first-order manner.

Conclusion

The configuration of D-galactose and the results of substrate testing have made possible a model for predicting other polyol substrates and their oxidation products from reaction with galactose

added to the reaction mixture prior to addition of galactose oxidase to prevent deactivation by H_2O_2. The reaction was allowed to continue at 4 ^{o}C for four days before any product determinations were made. The solution was heated and centrifuged to remove the enzymes.

An assay solution was prepared which contained the following: 0.66 g glycine and 1.47 mL hydrazine in 20 mL of water at pH 9.0. This solution was used for the assay of aldehyde and both lactate dehydrogenases.

The product solution was first checked with an aldehyde dehydrogenase assay to determine if the subject compound was a substrate of galactose oxidase. A typical assay contained 1.3 mL of the assay solution, 20 µL of the reaction solution, 20 µL of a 20 mg/mL NAD^+ solution, and 1.0 mL of H_2O. The assay was initiated by adding 20 µL of aldehyde dehydrogenase (4.0 mg/mL) to the solution, and the increase in absorbance at 340 nm due to the reduction of NAD^+ was observed.

The product solution was then allowed to react with the aldehyde dehydrogenase to give the carboxylic acid. Again the solution was heated and centrifuged to remove the enzyme. Aliquots of this mixture could then be assayed with L- and D-lactate dehydrogenase to determine optical purity according to the procedures reported previously (49).

A typical assay contained 100 µL NAD^+ (0.1 M), 2.7 mL glycine/hydrazine buffer, and 100 µL L-lactate dehydrogenase (0.5 mg/mL). The assay was initiated by adding 100 µL of the product solution, and the change in absorbance at 340 nm was monitored. The assay was then repeated under the same conditions with D-lactate

The following chemicals were purchased from Aldrich and used without further purification: (S)-1,2-propanediol, erythritol, (S)-1,3-butanediol, (R)-1,3-butanediol, galactitol, and xylitol. The following chemicals were purchased from Sigma and used without further purification: propylene glycol, DL-α-O-benzyl glycerol, DL-2-amino-1,3-propanediol, L-threitol, 2,3-O-isopropylidene-D-threitol, L-lyxose, D-arabitol, L-xylose, L-galactose, D-glyceraldehyde, D-arabinose, D-gulono-1,4-lactone, and D-ribose. D-Glucose and glycerol were obtained from Matheson Coleman & Bell, and D-fructose was obtained from Fischer. The 3-halo-1,2-propanediols and 3-butene-1,2-diol were all prepared by acid-catalyzed ring opening of the respective epoxides from Aldrich. (R)-1,2-Propanediol was prepared from L-threonine via formation of D- and L-lactaldehyde and subsequent reduction with $NaBH_4$ (47). D-Threitol was obtained by acid hydrolysis of its 2,3-isopropylidene derivative. 2-Methyl-1,3-propanediol was prepared by $NaBH_4$ reduction of diethylmethylmalonate. L-Glucitol was prepared by $NaBH_4$ reduction of D-gulono-1,4-lactone according to the literature method (48). All other chemicals were analytical grade.

Determination of Product Stereochemistry

The oxidation of glycerol, 3-chloro-1,2-propanediol, DL-α-O-benzyl glycerol, and the attempted oxidation of propylene glycol, (S)-1,3-butanediol, and (R)-1,3-butanediol were carried out by the method of Klibanov (33). Approximately 1 mg (25 units) of galactose oxidase was added to 0.5 mL of the subject compound at 2 M concentration in a 0.1 M phosphate buffer, pH 7.0. One mg (1600 units) of catalase was

CHAPTER VI

EXPERIMENTAL

General

All assays were taken on a Beckman DU-6 UV/Visible Spectrophotometer. Natural abundance ^{13}C NMR spectra were measured by using a JEOL PS100-FT spectrophotometer. Optical rotations were measured by using a Perkin-Elmer Model 241 polarimeter. HPLC analysis was performed by using a Waters μ-Bondapak carbohydrate column (0.4 x 30 cm) with refractometer detection and aqueous acetonitrile (CH_3CN/H_2O, 85:15, v/v) as solvent, flow rate 2 mL/min.

Materials

All enzymes were obtained from Sigma and used without further purification. Reported activities of the enzymes were as follows: galactose oxidase (D-galactose: O_2 oxidoreductase, EC 1.1.3.9), 25 units/mg solid; catalase (H_2O_2: H_2O_2 oxidoreductase, 1.11.1.6), 1600 units/mg solid; aldehyde dehydrogenase (aldehyde: $NAD(P)^+$ oxidoreductase, EC 1.2.1.5), 14 units/mg solid; alcohol dehydrogenase (alcohol: NAD^+ oxidoreductase, EC 1.1.1.1), 340 units/mg solid; L-lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase, EC 1.1.1.27), 920 units/mg protein (10.5 mg protein/mL); D-lactate dehydrogenase (D-lactate: NAD^+ oxidoreductase, EC 1.11.1.28), 14 units/mg solid; and peroxidase (Donor: H_2O_2 oxidoreductase, EC 1.11.1.7), 90 units/mg solid.

were obtained in three of the four reactions. Of these, the best explanation in the case of galactitol and L-glucitol seems to be slow inactivation of galactose oxidase by ferricyanide. The possibility that some ferrocyanide might be produced in the reaction certainly exists, but seems less likely. Product inhibition cannot be ruled out, although Montgomery and co-workers report that addition of fresh enzyme to the reaction mixture does not significantly increase the yield (26). Reaction conditions could also play a part in the yield, but overall, it would seem to be a combination of these reasons which prevent the reaction from going to completion.

of the detector. For this reason it seems more likely that the $K_3Fe(CN)_6$ inactivation of the enzyme is the best explanation of the low yield.

The D-threitol reaction did not have ferricyanide in the mixture, and yet still had a very low yield. This could have been caused by the reaction conditions. The reaction was run at room temperature in the hopes of increasing the reaction rate, but at the expense of denaturing the enzyme within a few days. Klibanov ran his experiments at 4 ^{o}C and did not observe that the reaction was complete for 21 days (33). He did not report any denaturation of the enzyme. Klibanov also had a very low yield, about 5%, (33). It could be that the enzyme reaction just does not carry through to completion. In fact, it has been reported that galactose oxidase reactions rarely go through to completion, even with the best substrates (26).

Attempts to determine the Michaelis constants of the substrates proved difficult, particularly using the peroxidase-chromagen assay. As was reported by Ettinger and Kosman (18), the reactions with ferricyanide did not exhibit saturation kinetics. Instead, a first-order rate dependence on the concentration of substrate was observed. The K_m of xylitol is so impossibly large (on the order of 20 M) that it cannot be approached because of the solubility of xylitol in water. The same applies to D-threitol. Since the Michaelis constants are so large, the reactions could not even approach one-half V_{max}.

Conclusion

There are a number of reasons which could explain why poor yields

greatly inhibit the reaction, and higher concentrations have an accelerating effect, or these results constitute an artifact as a result of the inadequate assay method used. No definitive statement can be made about the possibility of product inhibition with the current results.

The most likely reason for the low yield is inhibition by one of the reactants. The alditols probably are not responsible for the inhibition since a definite increase in reaction rate is observed with increasing concentrations of substrate. Oxygen is not an inhibitor as was shown by Hamilton and co-workers (38). Catalase, while lengthening the induction period somewhat, actually stabilizes the reaction (38). The only other reactant present is $K_3Fe(CN)_6$, which has been shown to inhibit galactose oxidase when present at concentrations of less than 0.001 m*M* (38). However, at the concentration used in these experiments, 0.050 m*M*, ferricyanide has a considerable activating effect.

Hamilton *et al.* reported a slow permanent inactivation of galactose oxidase by long-term contact with oxidants, to include ferricyanide (38). This inactivation could well account for the low yield in the two reactions in which it was present. In addition, the oxidation of the alditols by ferricyanide to the dicarboxylic acids, although extremely slow under reaction conditions, could generate enough ferrocyanide to inhibit the enzyme. Ferrocyanide has been determined to be a very potent inhibitor (38). It must be noted here that no side products were observed on the HPLC, so if ferricyanide oxidation of the alditols was occurring, it was below the sensitivity

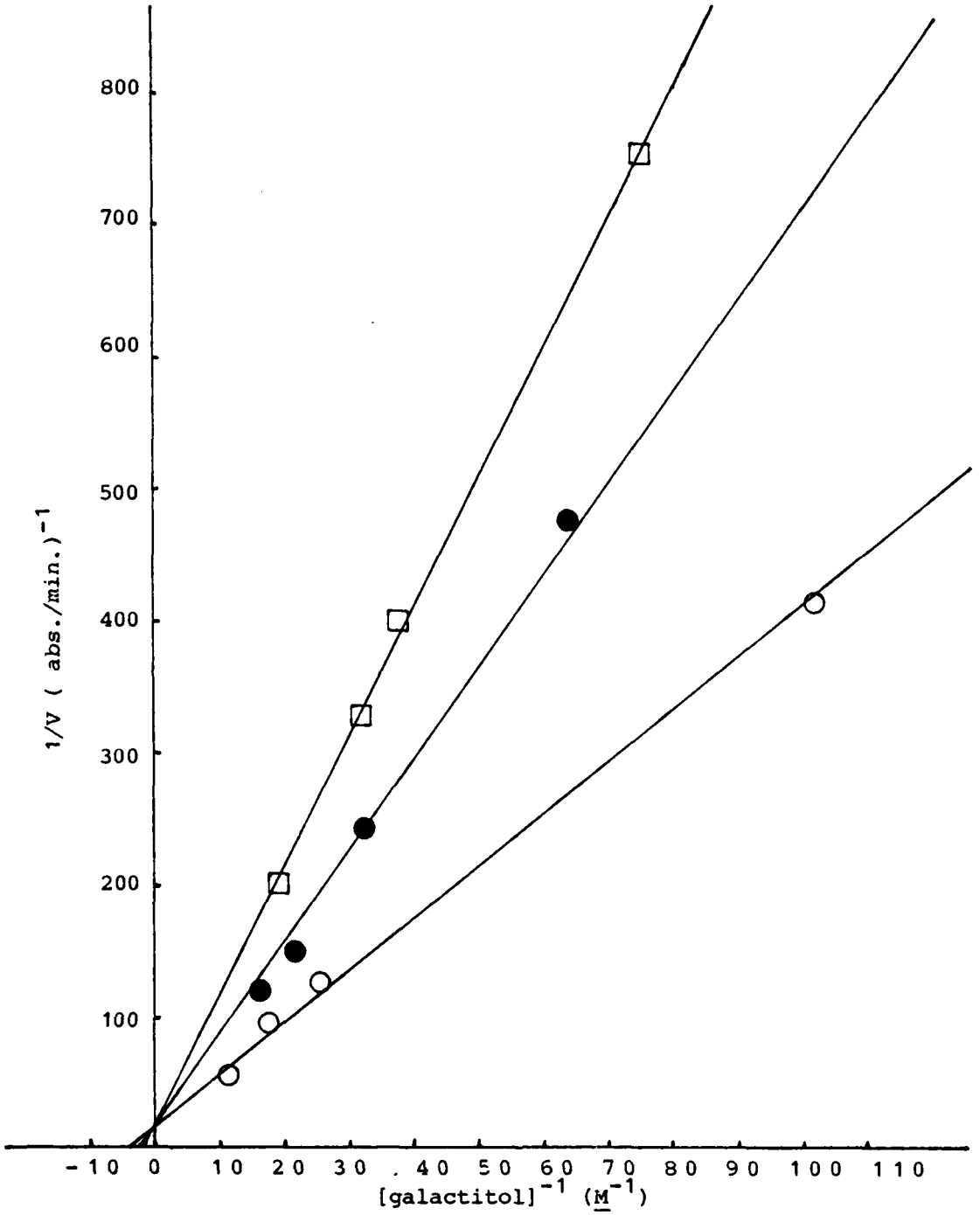


Figure 14. Inhibition studies of galactose oxidase oxidation of galactitol in the presence of L-galactose. Rates were measured at various galactitol concentrations at constant L-galactose concentrations of: 0.0 (○), 5.25 (□), and 71.2 (●) mM.

reduction of D-glucose indicates that the desired reaction might be extremely endothermic. Thus, based on the minimal data available, nothing certain can be asserted with respect to equilibria effects. It can be noted that one would expect the equilibria to lie more toward the product side since the products form very stable ring configurations.

Product inhibition could be handled in much the same way as an unfavorable equilibrium, assuming that the enzyme is not permanently inhibited by the product. Since the peroxidase-chromagen method used during this research cannot be used for detailed kinetic studies, only very simple and basic inhibition studies were attempted. Studies of the effect of the presence of L-xylose on the reaction of xylitol indicated no inhibition. However, studies of the effects of L-galactose on galactitol and of L-glucose on L-glucitol are less conclusive. In comparative assays, containing equal amounts of substrate, with only one containing product, the reaction with product appeared to proceed at a slightly faster rate. This was the case with both galactitol and L-glucitol. While admitting the inadequacy of the assay, an attempt was made to clarify the problem by varying amounts of product present with several different substrate concentrations. A graph of this was expected to show either some type of inhibition or activation. The results are shown in Figure 14. The reaction with a very small concentration, 5.25 m<u>M</u>, of product was greatly inhibited, but there was less inhibition when the product concentration was much higher, 71.2 m<u>M</u>. No explanation can currently be given to account for this apparent anomaly. Either very small concentrations of product

The change in free energy of the reaction, ΔG^{o}, can be estimated from:

$$\Delta G^{o} \approx \Sigma(\Delta G^{o}_{f\text{products}}) - \Sigma(\Delta G^{o}_{f\text{reactants}}). \qquad (2)$$

Since $\Delta G^{o} = -RT \ln K_{eq}$, then $K_{eq} = \exp(-\Delta G^{o}/RT)$, and $K_{eq} = 4.5 \times 10^{13}$. If the assumption in Equation 2 is valid, and these calculations are correct, the equilibrium lies very far to the product side. Values for free energies of formation were obtained from Stull, Westrum, and Sinke (44).

One might also get a qualitative value of the equilibrium constant from electrochemical data (45).

$$O_2 + 2H^{+} + 2e^{-} \longrightarrow H_2O_2 \qquad E^{o} = 0.682 \text{ V} \qquad (3)$$

$$\text{acetaldehyde} + 2H^{+} + 2e^{-} \longrightarrow \text{ethanol} \qquad E^{o} = -0.22 \text{ V} \qquad (4)$$

$$O_2 + \text{ethanol} \longrightarrow H_2O_2 + \text{acetaldehyde} \qquad E^{o} = 0.902 \text{ V} \qquad (5)$$

Subtracting Equation 4 from Equation 3 gives Equation 5. From the equations, $\Delta G = -nFE^{o}$ and $\Delta G = -RT \ln K_{eq}$ with n = 2 equivalents, F = 96490 coulomb/equivalent, and R = 8.3144 $J \cdot deg^{-1} \cdot mole^{-1}$ we obtain $K_{eq} = 3.2 \times 10^{30}$. Obviously, this is calculated with ethanol and acetaldehyde, but again the products would be anticipated to dominate.

A value for the change in enthalpy for the reduction of D-glucose to D-glucitol was found to be $\Delta H = -57.36$ kJ/mol (46). While not giving the free energy of the reaction, this data indicates that the L-glucitol-to-L-glucose reaction must be endothermic.

The K_{eq} calculated from ΔG^{o}_{f} or E^{o} is extremely high and favors formation of products. However, the reported enthalpy for the

Discussion

The results of these syntheses were generally poor, even though the substrates all appeared to have very good reaction rates in the assay. There are several possible explanations for the poor results. The most immediate which come to mind include equilibrium, product inhibition, inhibition by one of the reactants or catalysts, or other unforseen reaction or stability problems. If the problem is one of equilibrium or product inhibition, it can be easily solved. If the problem is one of the latter two mentioned above, better reaction conditions and/or methods of stabilizing the products in solution will have to be developed.

If the problem with the reaction is an equilibrium problem, it can be resolved by removing the product during the course of the reaction. The enzyme would have to be immobilized on a column, and the reaction mixture allowed to equilibrate on the column. The product can then be removed from the solution by passing through another column as reported by Wall and Jones (43). The separated starting material can then be recycled through the entire system.

While data is not available on the equilibrium constants of the reactions studied, the equilibrium constant of the galactitol/L-galactose reaction can be estimated from the difference in their free energies of formation:

$$\underset{\Delta G_f^o = -227.19 \text{ kcal/mol}}{\text{galactitol}} + O_2 \longrightarrow \underset{\Delta G_f^o = -217.62 \text{ kcal/mol}}{\text{L-galactose}} + \underset{\Delta G_f^o = -28.2 \text{ kcal/mol}}{H_2O_2}$$

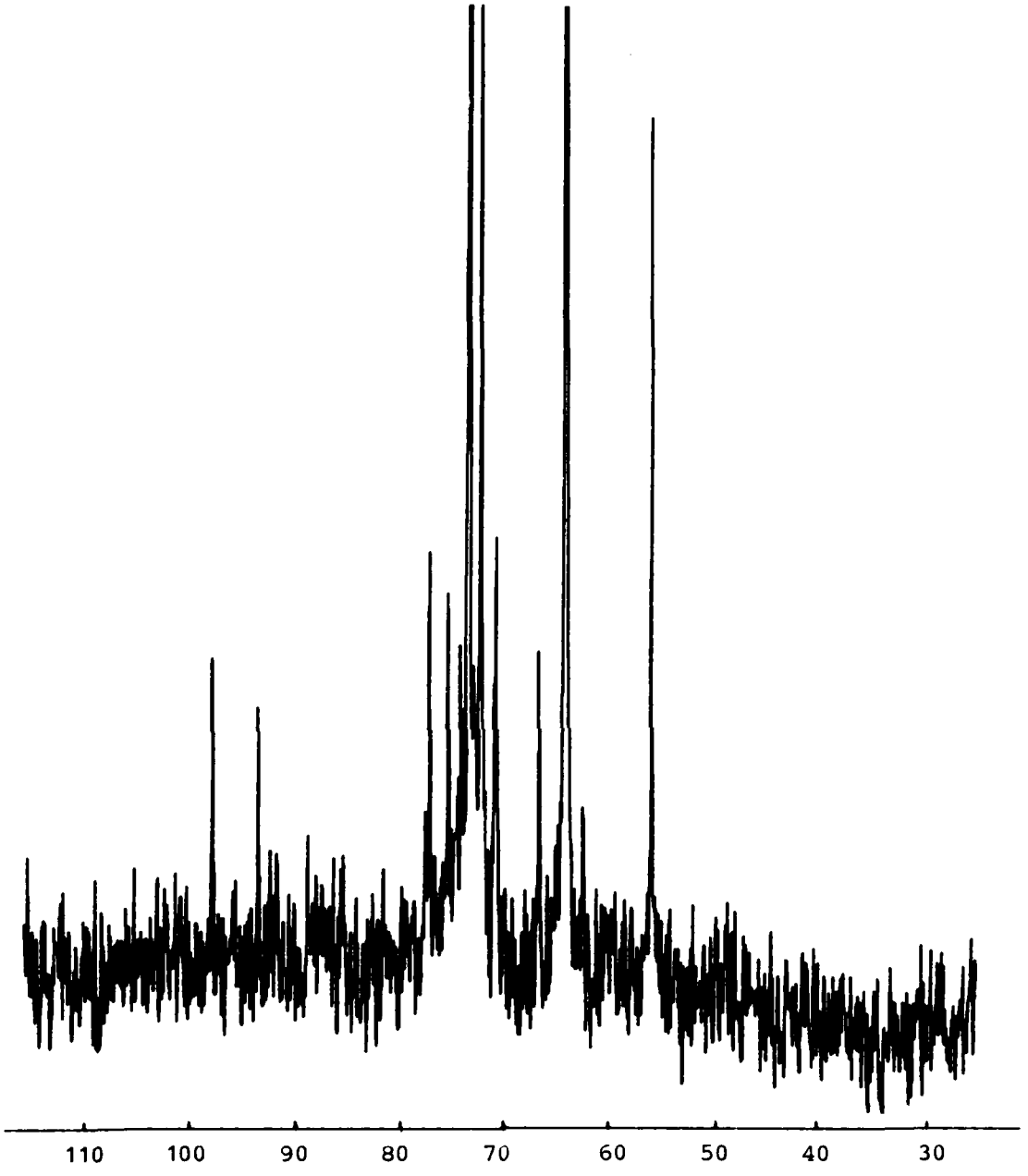


Figure 13. Natural abundance ^{13}C NMR spectrum of L-xylose/xylitol reaction mixture. L-Xylose: $\delta(D_2O)$ 97.6 (C-1β); 93.2 (C-1α); 76.8 (C-3β); 75.0 (C-2β); 73.8 (C-3α); 72.4 (C-2α); 70.4 (C-4α); 70.2 (C-4β); 66.2 (C-5β); 61.9 (C-5α). Xylitol: $\delta(D_2O)$ 72.9 (C-2); 71.7 (C-3); 63.6 (C-1). Note particularly the two characteristic hemiacetal peaks corresponding to the two anomers of L-xylose at δ:97.6 and 93.2 ppm downfield from DSS.

cofactors or donor compounds, was used in place of horseradish peroxidase.

Of the four reactions, only the synthesis of L-xylose proceeded with any substantial yield. The results of HPLC analyses for each of the reactions are given in Table 5. Polarimeter readings were taken for each of the reactions, but concentration mixtures were too small to give a measurable reading for D-threose, L-galactose, and L-galactitol. The specific rotation measured for L-xylose was $[\alpha]_D^{25}$ -17.6°, which was in agreement with the value reported previously, $[\alpha]_D^{24}$ -18.7° (c = 4, H_2O, 24 hr) (40). Natural abundance ^{13}C NMR spectra were taken for all reactions, but again, concentrations were too small to obtain visible results with D-threose, L-galactose, and L-glucose. The L-xylose spectra is shown in Figure 13. The spectrum is essentially the same as that reported in the literature (41,42).

TABLE 5

HPLC Analysis of Synthetic Reaction Products[a]

Reaction	Retention Time (min) Sugar Alcohol	Retention Time (min) Sugar	Approx. % Yield
18→62b	4.5	5.7	10
51→55	6.6	5.4	50
53→59	10.2	10.9	10
54→61	9.2	10.2	10

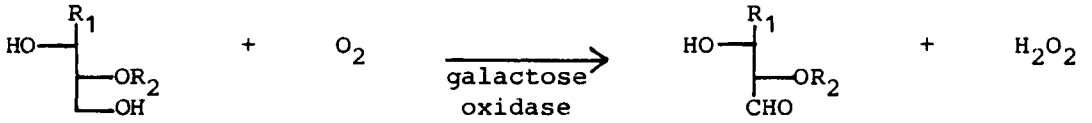
[a] Analysis was performed on a Waters μ-Bondapak carbohydrate column (0.4 x 30 cm), refractometer detection ($CH_3CN:H_2O$, 85:15), flow rate of 2 mL/min.

CHAPTER V

SYNTHESIS OF L-SUGARS USING GALACTOSE OXIDASE

Introduction

Since the continuing objective of this research is to develop new synthetic uses for enzymes, several of the polyol substrates identified in Chapter IV were used to attempt to synthesize and isolate their respective sugars. The substrates chosen for this effort and the expected products of their oxidation are shown in Figure 12.



50 62

50a = 18, 62a (D-threose): $R_1 = CH_2OH$; $R_2 = H$

50b = 51, 62b = 55: $R_1 = CHOHCH_2OH$; $R_2 = H$

50c = 53, 62c = 59: $R_1 = (CHOH)_2CH_2OH$; $R_2 = H$

50d = 54, 62d = 61: $R_1 = (CHOH)_2CH_2OH$; $R_2 = H$

Figure 12. Subject syntheses of unusual sugars.

Results

Because the observation of color change in the reacting solutions was not needed, the syntheses were conducted under slightly different conditions than the assays. Catalase (hydrogen-peroxide: hydrogen-peroxide oxidoreductase, EC 1.11.1.6), which does not require any

oxidase. Several of the compounds have been reported before, and their identification as substrates serves both to verify work done before and to act as a check on the validity of this research.

Polyols of three, four, five, and six carbons have been identified as substrates in agreement with the developed minimum structure. Three-, four-, and five-carbon polyols are very good substrates. Six-carbon polyols are very weak substrates presumably because of steric hindrance, but they may be enhanced to reasonable substrates by small concentrations of potassium ferricyanide.

dehydrogenase. The concentration of the D- or L-acid could be determined from the total change in absorbance and used in Equation 1 to calculate optical purity.

If the expected aldehyde product of the original oxidation was not a substrate of aldehyde dehydrogenase, optically pure starting materials were required. The denatured and centrifuged reaction solution (100 μL) was added to an assay solution containing 2.5 mL of glycine buffer (0.1 M, pH 9.0), 1 mg alcohol dehydrogenase, and 100 μL of a 0.1 M NADH solution. A decrease in absorbance at 340 nm indicated the presence of aldehyde. Concentrations calculated from the total change in absorbance from samples of the optically pure starting materials could then be used to calculate the enantiomeric excess of the reaction.

Substrate Identification

Further substrate identification was accomplished by using the horseradish peroxidase/o-dianisidine coupled assay of Bergmeyer (35). A reagent solution was prepared consisting of 10 mM phosphate buffer (pH 7.0), 0.2 mg/mL o-dianisidine, and 0.5 mg/mL peroxidase (45 units/mL). It was discovered that o-dianisidine is not very soluble in water, even at 0.2 mg/mL. The best results were obtained when the dye was first dissolved in methanol (5 mg/mL). The amount of methanol introduced into the assay by this was not enough to denature the enzyme, and methanol is not a substrate of galactose oxidase. In addition, o-dianisidine is a suspected carcinogen, so care was taken in its use.

A typical assay contained 1.5 mL of the reagent solution, enough substrate solution (less than 1.45 mL) to give a substrate concentration of 0.4 M or 50 mM, 50 μL galactose oxidase solution (0.5 mg/mL in doubly distilled water), and enough doubly distilled water to bring the total volume to 3.000 mL. Most compounds tested were assayed at 0.4 M or 50 mM in the interest of consistency and so that a comparison of substrate reactivity could be accomplished. In some cases, however, it was necessary to vary the compound concentration or the enzyme concentration due to availability of the compound or its reactivity with the enzyme.

Potassium ferricyanide was added to the assays of several of the weaker substrates to see if the rate could be increased. Since $K_3Fe(CN)_6$ absorbs strongly at 420 nm, the concentration was kept at 0.050 mM or less. Control assays were run to determine if the ferricyanide would react with any of the compounds in the assay without the presence of galactose oxidase.

Preparative Synthesis

The syntheses of D-threose and L-xylose were carried out under the following conditions. Catalase (3.6 mg, 5700 units) and galactose oxidase (0.3 mg, 6 units) were added to a 3.00 mL phosphate buffer solution (50 mM, pH 7.0) containing 50 mM substrate. The solution was allowed to react at room temperature for five days. The reaction was stopped and the enzymes precipitated by heating the vial in boiling water for 30 minutes. The solution was centrifuged, and the supernatant was recovered and concentrated under vacuum to a solid

residue. After HPLC analysis and ^{13}C NMR spectroscopy, the L-xylose/xylitol mixture was passed through an ion exchange column as described by Jones and Wall (43). Separation of the product from the starting material was confirmed by HPLC.

The syntheses of L-galactose and L-glucose were carried out under the following conditions. Catalase (2.2 mg, 3500 units) and galactose oxidase (6.5 mg, 160 units) were added to a 6.00 mL phosphate buffer solution (0.10 M, pH 7.0) containing 50 mM substrate and 0.050 mM $K_3Fe(CN)_6$. The rest of the procedure was as described for the syntheses of D-threose and L-xylose.

CHAPTER VII

CONCLUSION

The original objectives of this research were: to confirm the stereospecificity of galactose oxidase oxidation; to develop a minimum structure required for polyol substrates; to identify, using the model, polyols of three, four, five, and six carbons which are substrates of the enzyme; and to choose several substrates for the synthesis of their respective sugars.

The first objective was clearly met by the reaffirmation of Klibanov's results (33). Oxidation of glycerol, (R)-3-chloro-1,2-propanediol, and DL-α-O-benzyl glycerol yield L-glyceraldehyde, (R)-3-chloro-2-hydroxypropanal, and 3-O-benzyl-L-glyceraldehyde, respectively. In general, galactose oxidase will give aldehydes which are in the L-configuration at the carbon adjacent to the aldehyde.

Comparison of structures of already-known substrates of galactose oxidase and substrates identified by the assay of three- and four-carbon polyols led to the determination of a minimum required structure indicated in Figure 9. This structure was then used to predict five- and six-carbon polyols which might be substrates. The structure accurately predicted substrates, but could not take into account the steric hindrance caused by the active site ring-form preference.

Four of the substrates identified by assay were used to synthesize sugars. The results were very poor for three of the four substrates. L-Xylose, however, was synthesized in a fair yield.

Reasons for the poor results could not be determined with certainty. The equilibria would be expected to favor products, but estimated equilibrium constants were unusually large and appeared to contradict reported thermodynamic data. Inhibition studies gave conflicting results probably caused by the inadequacy of the assay method for the study of kinetic data. Other reasons for the poor results include slow inactivation of the enzyme by ferricyanide and possibly the denaturation of the enzyme at room temperature.

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VITA

Robert Lee Root was born 23 September 1957 in Lakeland, Florida to Robert Russell and Ruby Lee Root. Although an "Army Brat" for his first nine years, he grew up and finished grade school in Houston, Texas, graduating from Northbrook Senior High School on 28 May 1975. He received and accepted an appointment to the United States Military Academy at West Point, graduating on 6 June 1979 with a Bachelor of Science degree with concentration of electives in nuclear engineering and chemistry. He received a commission in the United States Army Chemical Corps as a Second Lieutenant and has since been promoted to Captain. He married Robin Matthews on 22 August 1981. He will receive his Master of Science degree on 3 May 1985, and has been reassigned by the Army to teach chemistry at West Point.

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